

Rapamycin Exerts Antifungal Activity *In Vitro* and *In Vivo* against *Mucor circinelloides* via FKBP12-Dependent Inhibition of Tor

Robert J. Bastidas,^a Cecelia A. Shertz,^a Soo Chan Lee,^a Joseph Heitman,^{a,b,c} and Maria E. Cardenas^a

Departments of Molecular Genetics and Microbiology,^a Medicine,^b and Pharmacology and Cancer Biology,^c Duke University Medical Center, Durham, North Carolina, USA

The zygomycete *Mucor circinelloides* is an opportunistic fungal pathogen that commonly infects patients with malignancies, diabetes mellitus, and solid organ transplants. Despite the widespread use of antifungal therapy in the management of zygomycosis, the incidence of infections continues to rise among immunocompromised individuals. In this study, we established that the target and mechanism of antifungal action of the immunosuppressant rapamycin in *M. circinelloides* are mediated via conserved complexes with FKBP12 and a Tor homolog. We found that spontaneous mutations that disrupted conserved residues in FKBP12 conferred rapamycin and FK506 resistance. Disruption of the FKBP12-encoding gene, *fkpA*, also conferred rapamycin and FK506 resistance. Expression of *M. circinelloides* FKBP12 (McFKBP12) complemented a *Saccharomyces cerevisiae* mutant strain lacking FKBP12 to restore rapamycin sensitivity. Expression of the McTor FKBP12-rapamycin binding (FRB) domain conferred rapamycin resistance in *S. cerevisiae*, and McFKBP12 interacted in a rapamycin-dependent fashion with the McTor FRB domain in a yeast two-hybrid assay, validating McFKBP12 and McTor as conserved targets of rapamycin. We showed that *in vitro*, rapamycin exhibited potent growth inhibitory activity against *M. circinelloides*. In a *Galleria mellonella* model of systemic mucormycosis, rapamycin improved survival by 50%, suggesting that rapamycin and nonimmunosuppressive analogs have the potential to be developed as novel antifungal therapies for treatment of patients with mucormycosis.

Comparative genomics to study closely related organisms has emerged as an important tool for understanding phenotypic differences, such as pathogenicity, and allows for identification of conserved molecular pathways that can be targeted in the development of broad-spectrum antimicrobial drugs. Conserved protein kinases controlling growth and proliferation of fungal pathogens represent attractive drug targets, both because they are likely to be essential for fungal propagation and development and because these enzymes are likely to be conserved among fungal species, allowing the use of inhibitors of these kinases as broad-spectrum antimicrobial therapeutic agents.

Among the repertoire of conserved protein kinases essential for cellular physiology, the Tor protein kinase is the focus of intense investigation, given its role as a central element of nutrient-regulated cell growth pathways. In *Saccharomyces cerevisiae*, Tor inhibition by the natural product rapamycin elicits many of the cellular responses that are triggered by nutrient starvation, such as inhibition of protein synthesis, downregulation of amino acid permeases, protein degradation, autophagy, and cell cycle arrest (reviewed in reference 45). These effects are mediated largely by the activity of Tor in promoting the expression of genes encoding tRNAs, ribosomal proteins, rRNAs, and amino acid permeases and suppressing nitrogen catabolite repression and the amino acid general control response (4, 6, 10, 22, 29, 41). In *S. cerevisiae*, two Tor proteins, Tor1 and Tor2, form two distinct multiprotein complexes, named TORC1 and TORC2 (33, 57). Collectively, these complexes control protein synthesis, mRNA synthesis and degradation, ribosome biogenesis, nutrient transport, and autophagy (TORC1), as well as actin polarization and cell wall integrity (TORC2) (reviewed in reference 60).

The Tor kinase has also received wide attention as an antifungal target due to its inhibition by the natural product rapamycin. Rapamycin was first identified as an antimicrobial with potent activity against *Candida albicans* (1, 56). Subsequently, rapamycin was shown to have robust antifungal activity against several hu-

man fungal pathogens, such as *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Fusarium oxysporum*, and several pathogenic *Penicillium* species (13, 58), and it was later found to have potent immunosuppressive activity (35). In yeast and mammalian cells, rapamycin inhibits Tor through its association with the prolyl isomerase FKBP12, forming a binary complex that binds to the highly conserved FRB (FKBP12-rapamycin binding) domain of Tor. This mechanism of action is supported by the identification of mutations in the FRB domain that confer rapamycin resistance by blocking FKBP12-rapamycin binding to Tor and by structural studies defining the molecular details of FKBP12-rapamycin inhibition of Tor (5, 11, 23, 34, 52, 59).

FKBP12 catalyzes *cis-trans* peptidyl-prolyl isomerization, a rate-limiting step in protein folding (reviewed in reference 27). Remarkably, FKBP12 also serves as the receptor for the antimicrobial and immunosuppressive drug FK506. Both rapamycin and FK506 bind to the active site of FKBP12 and inhibit its prolyl isomerase activity. The target of FKBP12-FK506 is calcineurin, a Ca²⁺-calmodulin-regulated serine-threonine-specific protein phosphatase consisting of a catalytic A (CnA) and a regulatory B (CnB) subunit (32). In humans, calcineurin regulates nuclear localization of the transcription factor NFAT during the response to antigen presentation (32). In *S. cerevisiae*, calcineurin regulates cation homeostasis and cell integrity via the transcription factor Crz1 (46). In *C. neoformans*, mutants lacking calcineurin are via-

Received 2 November 2011 Accepted 22 December 2011

Published ahead of print 30 December 2011

Address correspondence to Maria E. Cardenas, card004@mc.duke.edu.

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doi:10.1128/EC.05284-11

TABLE 1 Strains used in this study

Strain	Genotype	Reference or source ^a
<i>Phycomyces blakesleeanus</i> strain NRRL1555 (–)	Wild type	Santiago Torres-Martinez
<i>Rhizopus oryzae</i> strain FGSC9543	Wild type	Santiago Torres-Martinez
<i>Mucor circinelloides</i> f. <i>lusitanicus</i> strains ^b		
R7B (–)	<i>leuA</i> [–] (–)	Santiago Torres-Martinez
NRRL3631 (+)	Wild type (+)	Santiago Torres-Martinez
SM2	NRRL3631 (+) <i>fkBA-1</i> (A316G)	This study
SM4	R7B (–) <i>fkBA-2</i> (L91P)	This study
MU402	R7B (–) <i>pyrG</i> [–]	36
RBM1	MU402 <i>fkBA::pyrG</i>	This study
RBM2	MU402 <i>fkBA::pyrG</i>	This study
<i>Saccharomyces cerevisiae</i> strains		
JK9-3da	<i>MATa his4 HMLa leu2-3,112 rme1 trp1 ura3-52</i>	23
JHY3-3B	JK9-3da <i>fpr1::URA3</i>	23
CHY251	<i>MATa his3Δ200 leu2-3,112 trp1Δ101 ura3-52 vph6Δ::TRP1</i>	25
CHY516	<i>MATa his3Δ200 leu2-3,112 trp1Δ101 ura3-52 vph6Δ::TRP1 fpr1::ADE2</i>	25
SMY4-1	<i>MATa trp1-901 his3 leu2-3,112 ura3-52 ade2 gal4 gal80 URA3::GAL-lacZ</i> <i>LYS2::GAL-HIS3 TOR1-3 fpr1::ADE2</i>	34

^a The indicated strains were kindly provided by Santiago Torres-Martinez, Departamento de Genética y Microbiología, Facultad de Biología, Universidad de Murcia, Murcia, Spain.

^b Mating type for *Mucor* strains is indicated by “–” or “+”.

ble at 24°C but inviable at 37°C, and they are avirulent in animal models of cryptococcosis (18, 39).

While the mechanism of rapamycin action has been conserved in both ascomycete and basidiomycete fungal species (3, 13, 15), little is known about its activity in basal fungal lineages, such as the zygomycetes. One group within the zygomycetes is the order Mucorales, which arose early during fungal radiation and has emerged as an increasingly important cause of infection in humans. The majority of zygomycete infections are caused by *Rhizopus*, *Mucor*, *Rhizomucor*, *Cunninghamella*, and *Absidia* species and are prevalent among patients with diabetes mellitus and neutropenia and also among organ and hematopoietic stem cell transplant recipients (43, 50). Mortality rates of zygomycosis (also referred to as mucormycosis) can exceed 65% and 90%, especially among transplant patients, with an estimated annual incidence of 1.7 infections per million individuals in the United States alone (42, 51). Infections are acquired by inhalation of infectious spores from soil or dust and, less frequently, through breaches or injuries to the skin. Clinical manifestations of zygomycosis include severe necrosis of nasal, facial, and subcutaneous tissues, as well as rhinocerebral, pulmonary, and disseminated disease (44).

Treatment of zygomycosis relies on early detection of infection, surgical removal (debridement) of necrotic tissue, and aggressive antifungal therapy. Primary antifungal therapy has relied mostly on monotherapy with polyenes and lipid formulations of amphotericin B. Whereas most azoles, including fluconazole, voriconazole, and itraconazole, exhibit unreliable activity against zygomycosis, posaconazole has emerged as an option for salvage therapy in patients who are refractory to polyene treatment (44, 51, 53). Despite aggressive surgical and antifungal therapies for treatment of zygomycosis, the mortality rate associated with infections by these pathogens has remained high, warranting novel strategies for the treatment of zygomycosis.

Here we identify the FKBP12 and Tor homologs from *Mucor*

circinelloides, a common etiological agent of human zygomycosis. We found that rapamycin's antifungal action is mediated via FKBP12-rapamycin inhibition of Tor, based on the isolation and characterization of spontaneous FKBP12 mutations that confer resistance to rapamycin. We also demonstrate that *M. circinelloides* Tor (McTor) and FKBP12 interact in the presence of rapamycin and that disruption of the FKBP12-encoding gene, *fkBA*, confers rapamycin and FK506 resistance. Finally, we show that rapamycin treatment improves survival of the invertebrate *Galleria mellonella* in a model of systemic mucormycosis, suggesting a therapeutic effect of rapamycin. In summary, this study demonstrates the high conservation of rapamycin action among fungal pathogens, providing insights for novel therapeutic vantage points.

MATERIALS AND METHODS

Strains, media, drugs, and growth conditions. Strains used in this study are listed in Table 1. Strains were grown at 30°C or room temperature on YPD (1% yeast extract, 2% Bacto peptone, and 2% dextrose), potato dextrose agar (PDA), and RPMI (RPMI 1640 liquid medium with glutamine and without sodium bicarbonate [Gibco], supplemented with 2% dextrose and buffered with 0.165 M MOPS [morpholinepropanesulfonic acid] [pH 7]) media. Strains were grown either on solid media containing 2% agar or in liquid cultures. Rapamycin (LC Laboratories) was dissolved in 90% ethanol-10% Tween 20. FK506 (Prograph) was obtained from the Duke University Medical Center pharmacy. Rapamycin and FK506 stock solutions were added to media at the indicated concentrations.

Antifungal drug testing. Drug interactions were assessed by a checkerboard titration following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) (11a) for antifungal susceptibility testing of molds. *In vitro* testing was performed in RPMI 1640 medium. Aliquots of 50 μ l of each drug at a 4 \times concentration were dispensed to wells of a 96-well microtiter plate to provide 77 drug combinations. Additional rows were used to determine the MIC of each agent alone and for the growth control well (drug-free). Final drug concentrations tested were as follows: rapamycin, 200 μ g/ml to 3.12 μ g/ml (6 dilutions); and FK506,

25.0 $\mu\text{g/ml}$ to 0.1 $\mu\text{g/ml}$ (9 dilutions). To each well, an inoculum of 5×10^4 sporangiospores/ml was added, and microtiter plates were incubated at 35°C without shaking for 24 h. Growth inhibition determinations were performed with the aid of a concave mirror. The MIC and MIC₈₀ of drugs were defined as the lowest drug concentrations in the wells producing a complete absence of visual growth and an 80% reduction of growth, respectively.

Identification of *M. circinelloides* FKBP and Tor homologs. *M. circinelloides* FK506 binding proteins were identified by searching the *Mucor circinelloides* CBS277.49 genome database (<http://genome.jgi-psf.org/Mucci2/Mucci2.home.html>) for genes encoding proteins containing predicted FK506 binding domains with high homology to the FK506 binding domain of *S. cerevisiae* FKBP12 (ScFKBP12; encoded by *FPR1*) (see Fig. 2). Percent identity and similarity were determined by BLAST protein sequence alignments utilizing bl2seq from NCBI. Protein domains were identified using the Prosite database of protein domains, families, and functional sites (<http://prosite.expasy.org/>). The Tor homolog was identified by searching the *Mucor circinelloides* CBS277.49 genome database for proteins containing a Tor FRB domain with high homology to the *S. cerevisiae* Tor FRB domain. Phylogenetic reconstructions were performed with the Geneious (Biomatters) platform for sequence alignment, utilizing the Blossum 62 cost matrix and the Jukes-Cantor method for genetic distance modeling.

Plasmid construction. The *fkba* open reading frame was PCR amplified from an R7B cDNA library by use of primers containing a hemagglutinin (HA) epitope sequence (directly upstream of the *fkba* coding sequence) and PstI and SalI restriction sites. The amplicon was digested with PstI and SalI and cloned into PstI/SalI-digested pCu415CUP1 (30) to generate plasmid pCu415-HA-FKBP12. The FRB domain-encoding region of *torA* was amplified from R7B genomic DNA by use of primers containing a Myc epitope sequence (directly upstream of the FRB domain sequence) and EcoRI and PstI restriction sites. The amplicon was first digested with EcoRI, blunted with T4 DNA polymerase, and digested with PstI. The digested amplicon was cloned into SmaI/PstI-digested pCu414CUP1 (30) to generate plasmid pCu414-Myc-FRB. The yeast two-hybrid construct pGBKT7-FRB was generated by first amplifying the FRB domain sequence of *torA* from R7B genomic DNA by use of primers containing NcoI and BamHI restriction sites. The amplicon was then digested with NcoI and BamHI and ligated in frame with the GAL4 DNA binding domain (BD) into NcoI/BamHI-digested pGBKT7 (Clontech Laboratories Inc.). The pGADT7-FKBP12 plasmid was generated by amplifying the *fkba* coding sequence from pCu415-HA-FKBP12 by use of primers containing 30 bp of pGADT7 AD sequence (Clontech Laboratories Inc.) directly upstream of the EcoRI site (including the EcoRI site) and 30 bp of pGADT7 AD sequence directly downstream of the BamHI site (including the BamHI site). The amplicon was cotransformed with EcoRI-digested pGADT7 AD into *S. cerevisiae* strain SMY4-1 (Table 1) by a standard lithium acetate transformation method. Strains in which the *fkba* coding sequence was cloned in frame with the GAL4 activation domain (AD) in pGADT7 AD by homologous recombination were selected on complete synthetic medium lacking leucine (CSM–Leu). The presence of the cloned *fkba* coding sequence was confirmed by colony PCR. Total genomic DNA was extracted from positive transformants, and 2 μl of this DNA was transformed into *Escherichia coli* DH5 α cells (Invitrogen Corporation) to recover pGADT7-FKBP12 plasmids.

Yeast two-hybrid assay. The two-hybrid strain SMY4-1 (Table 1) was cotransformed with the two-hybrid fusion plasmids pGBKT7-FRB and pGADT7-FKBP12, cells were grown on CSM–Leu–Trp, and β -galactosidase activity was assayed using the chlorophenol- β -D-galactopyranoside (CPRG) substrate as previously described (8).

Molecular analysis of spontaneously drug-resistant *M. circinelloides* isolates. Spontaneously FK506 and rapamycin cross-resistant strains were isolated by spreading 500 *M. circinelloides* sporangiospores on YPD solid medium containing 1 $\mu\text{g/ml}$ FK506 and passaging FK506-resistant mycelial outgrowths on FK506-containing solid medium until

homokaryotic mycelial growth was observed. Spore suspensions were collected and tested for rapamycin resistance on YPD solid medium containing 100 $\mu\text{g/ml}$ rapamycin. Total DNA was isolated from *M. circinelloides* mycelia germinated in liquid YPD medium at 30°C by harvesting mycelia over Miracloth (Calbiochem, La Jolla, CA) and vacuum drying mycelia by lyophilization. Dried mycelia were triturated with glass beads by manual agitation and resuspended in 1 ml CTAB extraction buffer (100 mM Tris-HCl [pH 8.4], 1.4 M NaCl, 25 mM EDTA, 2% cetyltrimethylammonium bromide [CTAB]). A standard phenol-chloroform extraction procedure was used, followed by DNA precipitation using a 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. The DNA pellet was resuspended in 1 ml of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0). Genomic DNA was used to amplify 700-bp fragments by PCR, including 1-kb sequences upstream and downstream of the *fkba* coding sequence, and each fragment was sequenced. DNA analysis was compared by genomic DNA BLAST searches against the *M. circinelloides* genomic database sequences.

Gene expression analyses. *M. circinelloides* cultures were grown in liquid YPD medium at 30°C, and mycelium powder extracts were prepared as described above. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and 30 μg of total RNA was loaded onto a 1% formaldehyde agarose gel. Following transfer, membranes were hybridized to radioactive *fkba* and *actA* DNA-based probes. Hybridized probe signals were detected using a phosphorimager. cDNA was synthesized from 1.5 μg of total RNA by use of an AffinityScript multiple-temperature reverse transcriptase kit (Stratagene).

Western blot analyses. *M. circinelloides* protein extracts were generated from cultures grown in YPD medium at 30°C by harvesting mycelia over Miracloth (Calbiochem, La Jolla, CA) and vacuum drying mycelia by lyophilization. Dried mycelia were triturated with glass beads by manual agitation, and 20 mg was resuspended in 200 μl of sample buffer (1% SDS, 9 M urea, 25 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.7 M beta-mercaptoethanol) supplemented with phenylmethylsulfonyl fluoride (PMSF) and the indicated concentration of Roche Complete EDTA-free protease inhibitor cocktail. Samples were boiled for 2 min, vortexed for 1 min, and boiled again for 1 min. An additional centrifugation step was carried out for 15 min at 16,000 rpm, and supernatants were collected. For Western blotting, 0.3 mg of total protein extract was loaded on a 4 to 20% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ). Blots were blocked with 5% nonfat milk-0.05% Tween 20 in Tris-buffered saline, and membranes were incubated overnight at 4°C with a 1:1,000 dilution of antiserum against ScFKBP12, raised in rabbits (7). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody was used as the secondary antibody, at a dilution of 1:2,000. Equal loading of samples was monitored by stripping and incubating membranes for 1 h with a 1:2,000 dilution of a rat anti- α -tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated anti-rat IgG antibody was used as the secondary antibody, at a 1:2,000 dilution. Proteins were visualized by using ECL Western blotting substrate (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol.

S. cerevisiae total protein extracts were prepared by disrupting cells with glass beads in lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 100 μM Na₃VO₄, 25 mM beta-glycerophosphate, 25 mM NaF) supplemented with PMSF and the indicated concentration of Roche Complete EDTA-free protease inhibitor cocktail. Fifty micrograms of total protein extract was used for Western blotting as described above.

Disruption of the *M. circinelloides* *fkba* gene. An R7B *fkba* disruption cassette was generated by overlap PCR amplification of an amplicon containing 1,000 bp of *fkba* genomic DNA sequence directly upstream of the ATG codon, with an added XbaI restriction site; a 2,000-bp *pyrG* gene amplicon; and an amplicon with 1,000 bp of *fkba* genomic DNA sequence directly downstream of the *fkba* coding sequence stop codon, with an added XmaI restriction site. The resulting *fkba::pyrG* disruption cassette

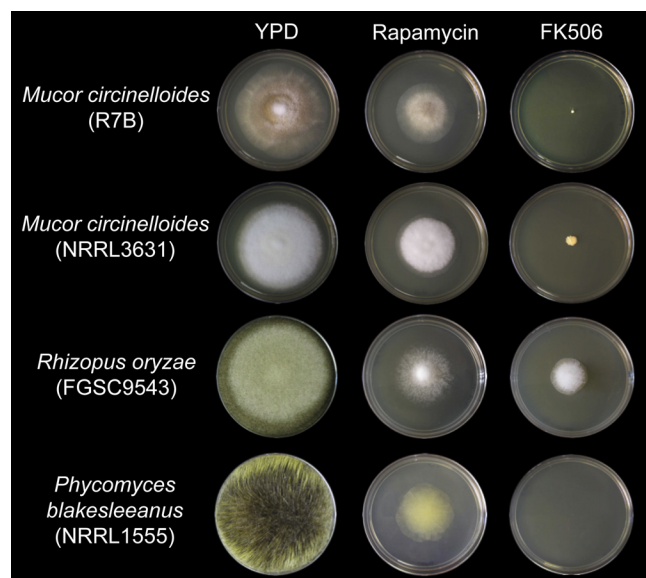


FIG 1 Growth of representative zygomycete species is compromised when organisms are exposed to rapamycin and FK506. Spores (500 spores per plate) from representative zygomycete species were germinated on YPD agar containing either 100 nM rapamycin or 1 µg/ml FK506. Spores were germinated for 72 h at room temperature.

was digested with XbaI and XmaI and ligated into pJAF12, yielding plasmid pJAF12/fkbA::pyrG. The disruption plasmid was linearized with XhoI and dephosphorylated with calf intestinal phosphatase (CIP; New England BioLabs), and 50 µg of digested plasmid was transformed into two independent MU402 (R7B *pyrG*[−] strain) (Table 1) cultures. Transformation was performed as described in reference 55, with a modified protoplast preparation step as described in reference 37, in which 2.5×10^8 germinated spores were incubated with 5 mg/ml chitosanase (molecular grade; U.S. Biologicals RD) and 0.5 mg/ml lysing enzymes (Sigma) for 90 min at 30°C. *fkbA* disruption was verified by PCR (see Fig. 6B) utilizing primers P1 (JOHE20967; 5'-GAAAATAAGAAAAGAATAAATTAGATTAC-3'), P2 (JOHE20968; 5'-AAGCACCTATTATATGGAGATAGAAC-3'), P3 (JOHE20965; 5'-AAAGTATATTAAAGGCAATAAAGTACAAT-3'), and P4 (JOHE20966; 5'-AATAGACAATAATTCTTTCTTTGTAGTAAAC-3') and by Southern blot analysis (data not shown).

Galleria mellonella model of systemic zygomycosis. *M. circinelloides* inoculums were prepared in phosphate-buffered saline (PBS) by suspending sporangiospores harvested from mycelial lawns grown on PDA for 5 days at room temperature, and spore titers were determined by hemocytometer counting. Drug stocks were prepared by dilution in 90% ethanol-10% Tween 20. Measurements of the hemolymph volume of 20 larvae of various weights and determination of the mean hemolymph volume/kg of body weight by linear regression analysis were used to determine the drug doses administered. Larvae in the final instar were obtained from Vanderhost, Inc. Ten larvae (300 mg \pm 25 mg) were used per group. Each larva was injected with 5 µl of PBS containing 500 sporangiospores, PBS only, drugs, or vehicle into the hemocoel via a proleg. At 4 h postinfection, 5 µl of PBS containing drugs or vehicle control was injected into each larva via a separate proleg from the one used during infection. Larvae were incubated at room temperature, and the number of dead larvae was scored daily. Kill curves were plotted, and differences in survival rates (log rank test) were determined by the Kaplan-Meier method, using GraphPad Prism software.

RESULTS

Growth of representative zygomycete species is sensitive to rapamycin. While the mechanisms of action of rapamycin (siroli-

TABLE 2 Radial colony diameters of zygomycete species grown in the presence of YPD, rapamycin, and FK506^a

Strain	Mean (SE) colony diam (cm) ^b		
	YPD	YPD + rapamycin	YPD + FK506
<i>Mucor circinelloides</i> R7B	6.8 (0.065)	5.0 (0.061)	0.4 (0.053)
<i>Mucor circinelloides</i> NRRL3631	7.2 (0.077)	4.9 (0.080)	0.6 (0.042)
<i>Rhizopus oryzae</i> FGSC9543	8 (0)	3.7 (0.086)	0.5 (0.030)
<i>Phycomyces blakesleeanus</i> NRRL1555	8 (0)	4.0 (0.061)	0 (0)

^a Spores (500 spores per plate) from representative zygomycete species were germinated on YPD agar containing either 100 nM rapamycin or 1 µg/ml FK506. Spores were germinated for 72 h at room temperature, and colony diameters were measured.

^b Standard errors were calculated for 8 replicates.

mus) and FK506 (tacrolimus) have been established for several ascomycetous and basidiomycetous fungal pathogens, little is known about the activities of these antifungal agents and their mechanisms of action in basal fungal lineages, which include several fungal pathogens of clinical and environmental importance.

To further assess the activities of these natural products among basal fungal species, growth assays of the zygomycete species *M. circinelloides*, *Rhizopus oryzae*, and *Phycomyces blakesleeanus* were performed in the presence of both rapamycin and FK506. Growth of *M. circinelloides* strains R7B (− mating type) and NRRL3631 (+ mating type) and of *R. oryzae* and *P. blakesleeanus* strains was evaluated on rich agar media containing rapamycin or FK506. While all four strains exhibited reduced radial mycelial growth in the presence of rapamycin, *P. blakesleeanus* growth exhibited the highest sensitivity to rapamycin, with a clear reduction in both radial mycelial growth and apical sporangiophore formation (Fig. 1 and Table 2). As observed with rapamycin, FK506 strongly inhibited the growth of all zygomycete species tested (Fig. 1 and Table 2).

In addition, we also determined the rapamycin and FK506 MICs for inhibition of growth of all three zygomycete species, following CLSI's guidelines for antifungal susceptibility testing of molds by broth microdilution (11a). We found that rapamycin inhibited up to 80% of growth at concentrations above 6.26 µg/ml, 12.5 µg/ml, and 100 µg/ml for *P. blakesleeanus*, *R. oryzae*, and *M. circinelloides*, respectively (Table 3). As expected, *P. blakesleeanus* growth inhibition required lower concentrations of rapamycin, while surprisingly, *M. circinelloides* growth inhibition required high concentrations of rapamycin during liquid culture growth (Table 3). Interestingly, growth of *R. oryzae* exhibited the least sensitivity to FK506 on agar medium containing FK506 (Fig.

TABLE 3 Rapamycin and FK506 MIC₈₀s during growth of zygomycete species

Strain	MIC ₈₀ (µg/ml) ^a	
	Rapamycin	FK506
<i>Mucor circinelloides</i> R7B (−)	100–200	0.8–1.6
<i>Mucor circinelloides</i> NRRL3631 (+)	100–200	0.4–0.8
<i>Rhizopus oryzae</i> FGSC9543	12.5–25	0.2–0.4
<i>Phycomyces blakesleeanus</i> NRRL1555	6.3–12.5	0.2–0.4

^a Drug dilutions tested ranged from 200 to 0.09 µg/ml.

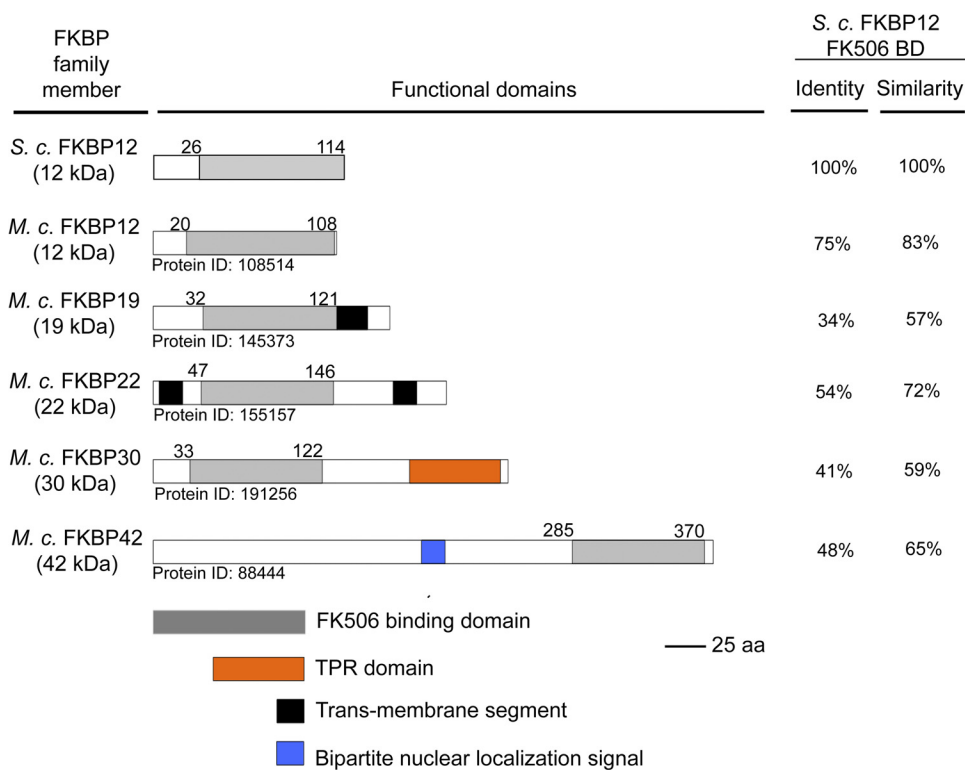


FIG 2 *M. circinelloides* family of FK506 binding proteins. Family members were identified based on their protein sequence homology to the FK506 binding domain of *S. cerevisiae* FKBP12. Identity and similarity scores correspond to similarities between the protein sequence of the FK506 binding domain of each *M. circinelloides* FKBP family member and the corresponding domain of *S. cerevisiae* FKBP12. The nomenclature for each FKBP (FK506 binding protein) family member is based on its predicted molecular mass (denoted in a parenthesis below each homolog). Protein IDs are from the *Mucor circinelloides* CBS277.49 v 2.0 genome database, hosted at the Joint Genome Institute (JGI) website. TPR, tetratricopeptide repeat.

1), while it exhibited a higher sensitivity to FK506 than those of the *Mucor* and *P. blakesleeanus* strains in a broth microdilution assay (Table 3). While the source of these discrepancies remains unknown, they are likely a result of differences in *R. oryzae* growth properties on semisolid surfaces and liquid medium. Nevertheless, both assays clearly indicate that *R. oryzae* is highly susceptible to FK506. In contrast to the fungicidal activity of rapamycin against the pathogens *C. albicans* and *C. neoformans* (MICs of <0.09 µg/ml and >0.19 µg/ml, respectively [15]), zygomycete growth was not entirely inhibited by rapamycin, and inhibition required significantly higher concentrations of drug, which could be due partly to decreased permeability of zygomycete cell walls and/or membranes to rapamycin or higher-affinity drug pumps or to occlusion of the FRB domain in zygomycete Tor homologs. Nevertheless, the effect of rapamycin on the growth of the tested zygomycete species provides evidence that in these organisms, Tor also functions to control growth. The MIC₈₀s of FK506 were significantly lower than those observed for rapamycin (Table 3), in agreement with the phenotypes observed during growth on FK506-containing agar medium. These results illustrate that FK506 exhibits potent antifungal activity against the zygomycete species tested.

The *M. circinelloides* FKBP12 homolog and the FRB domain of Tor are required for rapamycin action. We found that growth of *M. circinelloides* strains was compromised during rapamycin treatment. The presumed targets of rapamycin in this fungal species are the FKBP12 and Tor homologs. Comparative analysis of

the *M. circinelloides* genome identified a family of 5 genes encoding 5 putative proteins with closely related FK506 binding domains. We named these genes *fkba*, *fkbb*, *fkbc*, *fkbd*, and *fkbe* (FK506 binding), and based on the predicted molecular weights of the proteins they encode, we named their products FKBP12, FKBP19, FKBP22, FKBP30, and FKBP42, respectively (Fig. 2). FK506 binding proteins with various molecular weights are ubiquitous in nature, and members of the FKBP family have been identified in multiple organisms, including humans, plants, and fungi (40). FKBP12 family members contain only one FK506 binding domain, while FKBP3s with high molecular weights possess extra domains, such as tetratricopeptide repeat domains and calmodulin binding and transmembrane motifs (20). Based on the 12-kDa predicted molecular mass of the *M. circinelloides* putative homolog and on phylogenetic analyses of the McFKBP family of proteins and several fungal FKBP12 homologs (Fig. 3), we identified *fkba* as the gene encoding the McFKBP12 homolog.

To test whether the product of *fkba* functions as the rapamycin/FK506 receptor in *M. circinelloides*, we performed functional complementation studies using *S. cerevisiae* as a heterologous host. From an *M. circinelloides* (R7B) cDNA library, we amplified an HA-tagged *fkba* coding sequence amplicon and cloned it into a yeast low-copy-number plasmid under the control of an inducible copper promoter (pCu415CUP1) (30). The resulting plasmid (pCu415-HA-FKBP12) was introduced into a rapamycin-resistant *S. cerevisiae* strain lacking the FKBP12 homolog (*fpr1Δ*) (23) and tested on rapamycin-containing YPD agar plates with or

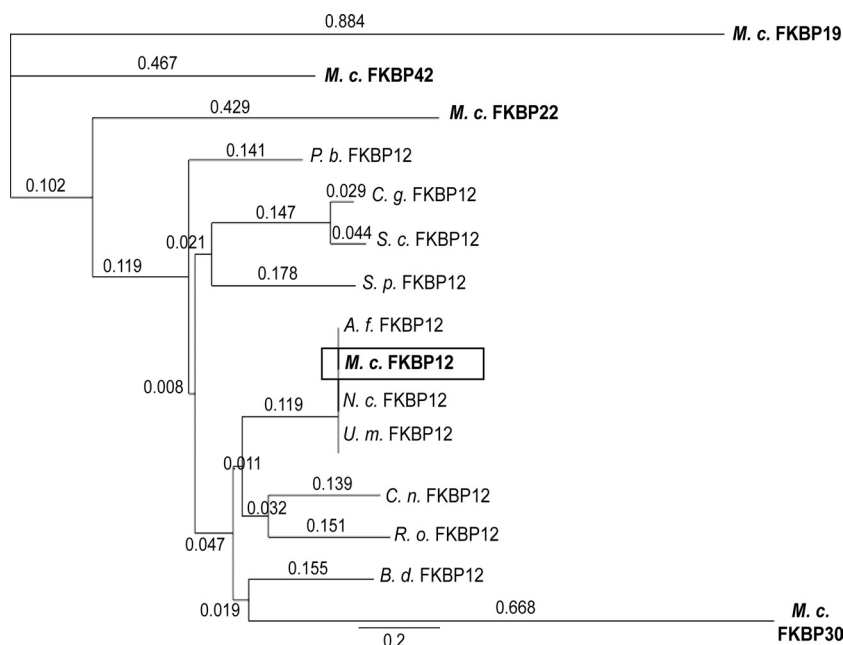


FIG 3 Phylogenetic reconstruction of *M. circinelloides* FKBP family of proteins and fungal FKBP12 homologs. The McFKBP12 homolog was identified by protein sequence comparisons with *Phycomyces blakesleeana* (*P. b.*), *Candida glabrata* (*C. g.*), *S. cerevisiae* (*S. c.*), *Schizosaccharomyces pombe* (*S. p.*), *Aspergillus fumigatus* (*A. f.*), *Neurospora crassa* (*N. c.*), *Ustilago maydis* (*U. m.*), *Cryptococcus neoformans* (*C. n.*), *Rhizopus oryzae* (*R. o.*), and *Batrachochytrium dendrobatidis* (*B. d.*) FKBP12 homologs.

without exogenous copper. Heterologous expression of *Mcfkba* restored rapamycin sensitivity in the *fpr1Δ* strain, indicating that McHA-FKBP12 can functionally complement an *S. cerevisiae* strain lacking endogenous FKBP12 (Fig. 4A).

We also tested whether McHA-FKBP12 can mediate FK506-dependent inhibition of *S. cerevisiae* calcineurin by transforming the *Mcfkba*-containing construct into an *S. cerevisiae vph6Δ fpr1Δ* double mutant strain (25). Loss of *VPH6* (which encodes a component of the vacuolar H⁺-ATPase assembly complex) results in a synthetic lethal phenotype during inhibition of calcineurin by FK506, whereas a *vph6Δ fpr1Δ* mutant strain also lacking FKBP12 is FK506 resistant (see Fig. S1A in the supplemental material) (24). Surprisingly, heterologous expression of McHA-FKBP12 did not render the *vph6Δ fpr1Δ* strain sensitive to FK506 (see Fig. S1), indicating that McHA-FKBP12 is unable to inhibit calcineurin function during FK506 exposure, even though the McHA-FKBP12 protein was stably expressed in a *vph6Δ fpr1Δ* background in a Western blot (data not shown). This may reflect differences in the composite FKBP12-FK506-calcineurin binding surface that arose during divergence of *S. cerevisiae* and *M. circinelloides* from their last common ancestor.

Genome analysis also revealed the presence of one gene encoding a Tor homolog (*Mucor circinelloides* CBS277.49 protein ID 152074), which we designated *torA*. The FRB domain of Tor was PCR amplified from *M. circinelloides* (R7B) genomic DNA and cloned into a yeast low-copy-number plasmid containing a copper-inducible promoter (pCu414CUP1) (30). A wild-type *S. cerevisiae* strain (JK9-3da) was transformed with this plasmid and tested on rapamycin-containing YPD agar in the presence and absence of exogenous copper. In the presence of copper, expression of the *M. circinelloides* FRB domain rescued the wild-type strain from the fungicidal activity of rapamycin, while a wild-type

strain transformed with vector alone remained sensitive to rapamycin (Fig. 4B). These results indicate that the *M. circinelloides* FRB domain can effectively compete for rapamycin binding (when overexpressed in the presence of copper) and is therefore the likely target of the FKBP12-rapamycin complex in *M. circinelloides*.

The yeast two-hybrid reporter system was used to determine whether McFKBP12 interacts with the FRB domain of McTor in the presence of rapamycin. To this end, a rapamycin-resistant yeast two-hybrid host strain containing a *TOR1-3* mutation and lacking endogenous FKBP12 was employed (SMY4-1) (8). This strain was transformed with plasmids expressing McFKBP12 fused to the GAL4 AD (pGADT7-FKBP12) and the McTor FRB domain fused to the GAL4 BD (pGBKT7-FRB), and interactions were quantified by monitoring expression of a *GAL4-lacZ* reporter gene. Strong interactions between FKBP12 and the FRB domain were observed only in the presence of rapamycin, indicating that the FRB domain of Tor is a target for rapamycin when Tor is complexed with FKBP12 (Fig. 4C). In summary, the McFKBP12 homolog can function as the receptor for rapamycin and can mediate inhibition of Tor via the FRB domain in a heterologous host.

Mutations in *fkba* confer rapamycin and FK506 resistance. The observation that McFKBP12 can mediate rapamycin inhibition of ScTor yet is unable to promote inhibition of *S. cerevisiae* calcineurin in the presence of rapamycin prompted us to employ a genetic approach to assess whether McFKBP12 functions as the rapamycin/FK506 receptor in *M. circinelloides*. Exploiting the robust antifungal activity of FK506 toward *M. circinelloides* (Fig. 1 and Tables 2 and 3), we isolated a series of strains in two independent strain backgrounds, R7B (– mating type) and NRRL3631 (+ mating type), that exhibited spontaneous FK506 resistance, and we examined the *fkba* locus for the presence of genetic lesions.

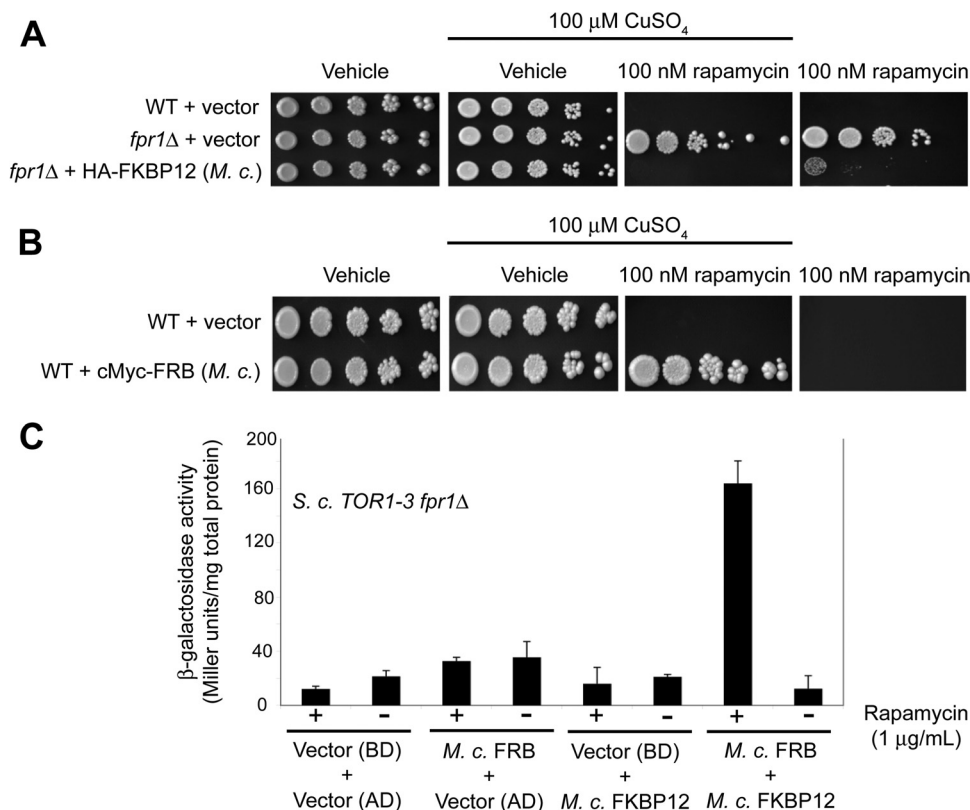


FIG 4 FKBP12 and the Tor FRB domain of *M. circinelloides* interact and can functionally complement *S. cerevisiae* wild-type and *fpr1* Δ strains. (A) McFKBP12 mediates inhibition of ScTor by rapamycin in an *S. cerevisiae* *fpr1* Δ strain lacking FKBP12. Wild-type (JK9-3da) cells transformed with vector alone (pCu415CUP1 [*LEU2*]) are sensitive to rapamycin, while *fpr1* Δ cells (JHY3-3B) transformed with vector alone (pCu415Cup1) are resistant. Expression of *McHA-FKBP12* (pCu415-HA-FKBP12) renders an *fpr1* Δ strain (JHY3-3B) sensitive to rapamycin. (B) Overexpression of the FRB domain of *McTor* (pCu414-cMyc FRB) in a wild-type strain (JK9-3da) competes with endogenous Tor1/Tor2 for rapamycin binding, suppressing the fungicidal activity of rapamycin observed in a wild-type (JK9-3da) strain transformed with vector alone (pCu414CUP1 [*TRP1*]). (C) The FRB domain of *McTor* and *McFKBP12* interact in the presence of rapamycin in the rapamycin-resistant yeast two-hybrid host SMY4-1 (*TOR1-3 fpr1* Δ *GAL4-lacZ*). SMY4-1 strains transformed with vector alone (pGADT7AD or pGBKT7) or with vectors expressing the FRB domain fused to the GAL4 DB (pGBKT7-FRB) or FKBP12 fused to the GAL4 AD (pGADT7AD-*fkba*) fail to induce strong expression of the *GAL4-lacZ* reporter in the presence or absence of rapamycin. Results represent three independent replicates.

Two isolates were identified: one, SM2 (NRRL3631 background), was cross resistant to rapamycin and FK506, and a second isolate, SM4 (R7B background), was sensitive to rapamycin and resistant to FK506 (Fig. 5A). Both isolates were sensitive to the calcineurin inhibitor CsA, indicating that these are not multidrug-resistant isolates (Fig. 5A). SM2 contains an *fkba* allele (*fkba-1*) harboring an A-to-G substitution (A316G) in the acceptor splice site of intron 2. Amplification of the *fkba* coding domain sequence from a cDNA library synthesized from SM2 total RNA revealed the presence of several alternative *fkba* cDNA species of various sizes (Fig. 5B). Sequence analysis of the smallest species revealed a 28-bp deletion of exon 3 sequence due to the use of a cryptic splice site located downstream from the acceptor splice site in wild-type *fkba* (see Fig. S2 in the supplemental material). In two of the *fkba-1* mRNA species, intron 2 was retained due to the A316G substitution, leading to longer *fkba* cDNA species (see Fig. S2). For all three cDNA/mRNA species, the predicted proteins contain premature stop codons upstream of the FK506 binding domain (see Fig. S2), and no FKBP12 protein products were detectable by Western blotting (Fig. 5C).

The FK506-resistant isolate SM4 contains an *fkba* allele (*fkba-2*) in which a mutation in exon 3 results in a leucine-to-

proline substitution (L91P) in the predicted protein sequence of FKBP12 that does not affect FKBP12 protein production (Fig. 5C). Leucine 91 is a highly conserved residue among FKBP12 homologs (Fig. 5D) and has been identified as a critical residue required for binding of human FKBP12 to calcineurin (21). In SM4, the L91P substitution leads to a shorter cyclic side chain that would predictably diminish calcineurin binding in the presence of FK506 (see Discussion). Taken together, our genetic analysis strongly suggests that FKBP12 functions as the rapamycin/FK506 receptor in *M. circinelloides*.

Disruption of *fkba* by homologous recombination renders *M. circinelloides* resistant to rapamycin and FK506. To further establish that FKBP12 is the cognate receptor for rapamycin and FK506, we disrupted the *fkba* locus by replacing the coding domain sequence with a *pyrG* disruption cassette. A gene replacement allele comprised of the *pyrG* gene and 1-kb sequences from the *fkba* 5'- and 3'-untranslated regions (see Materials and Methods) was linearized and used to transform the uridine auxotrophic strain MU402 (*leuA*⁻ *pyrG*⁻) (36). Two independent transformations were performed to ensure that disruption of the *fkba* gene resulted from two independent events. PCR analysis of two independent homokaryotic uridine prototrophic transformants,

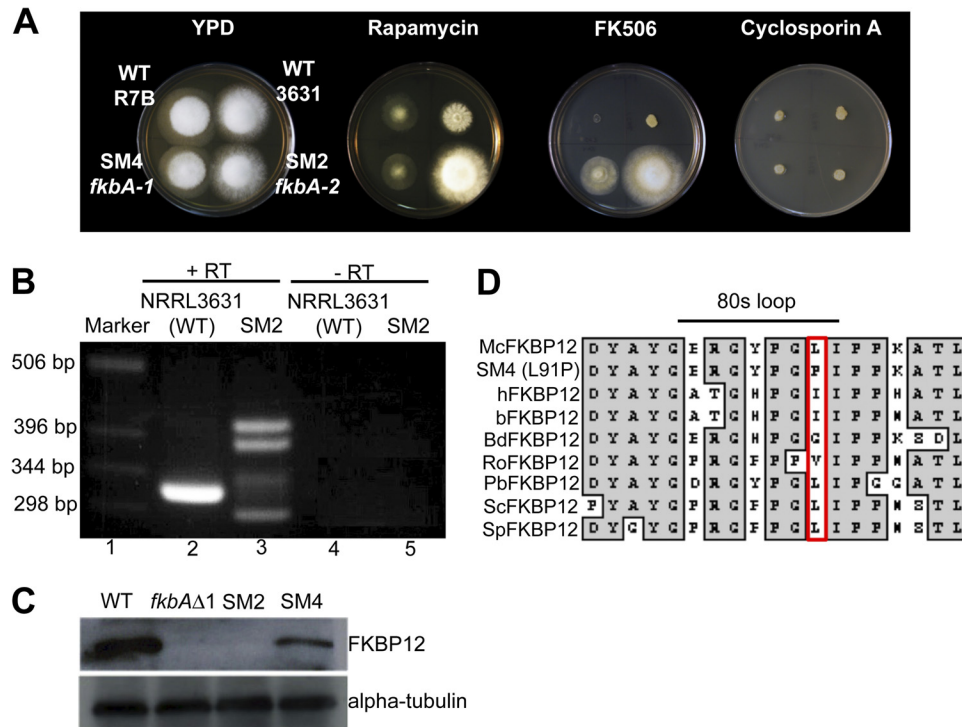


FIG 5 Spontaneous mutations in *fkbA* confer rapamycin and FK506 resistance. (A) Spores from strains R7B (WT $-$), NRRL3631 (WT $+$), SM4 [*fkbA-1*(L91P)], and SM2 [NRRL3631 *fkbA-2*(A316G)] were plated on YPD plates with or without 100 nM rapamycin, 1 μ g/ml FK506, or 100 μ g/ml cyclosporin A. SM2 is cross resistant to rapamycin and FK506, while SM4 is rapamycin sensitive and FK506 resistant. (B) An A316G substitution in the acceptor splice site of intron 2 in the *fkbA* locus in SM2 leads to the production of alternative *fkbA* cDNA species. Three *fkbA* alternative transcripts were amplified from an SM2 cDNA library (lane 3). The alternative cDNA species were not detected in no-reverse-transcriptase controls ($-$ RT). *fkbA-1* species a and b retain intron 2. *fkbA-1* species c harbors a 28-bp deletion in exon 3 due to the alternative use of a cryptic acceptor splice site upstream of the wild-type acceptor splice site (see Fig. S2 in the supplemental material). (C) SM2 does not express FKBP12. FKBP12 protein expression was determined by Western blot analysis of wild-type (R7B), *fkbAΔ1* (*fkbA* deletion strain [see Fig. 6]), SM2, and SM4 total lysates. FKBP12 was detected with antiserum raised against ScFKBP12 (see Materials and Methods). An *fkbAΔ* deletion strain was used as a control for FKBP12 antiserum specificity. Alpha-tubulin levels served as loading controls. (D) Protein alignments of the 80s loop in *Mucor circinelloides* (R7B) (Mc), SM4 (L91P), human (h), bovine (b), *Batrachochytrium dendrobatidis* (Bd), *Rhizopus oryzae* (Ro), *Phycomyces blakesleeanae* (Pb), *Saccharomyces cerevisiae* (Sc), and *Schizosaccharomyces pombe* (Sp) FKBP12 homologs. Leucine 91 in *M. circinelloides* and the L91P mutation in SM4 are boxed in red.

named RBM1 (*fkbAΔ1*) and RBM2 (*fkbAΔ2*), confirmed integration of the *pyrG* disruption allele at the *fkbA* locus (Fig. 6A and B). Disruption of *fkbA* in these two strains was also confirmed by Southern blot analysis (data not shown).

Northern analysis was further employed to confirm the loss of the *fkbA* gene. Employing a probe that hybridizes to exon 2 of *fkbA*, we were able to detect *fkbA* mRNA production in the wild-type parental strain (MU402) and failed to detect the message in each of the two independent *fkbAΔ* deletion strains (Fig. 6C). An antiserum raised against *S. cerevisiae* FKBP12 (7) was employed to confirm the loss of the FKBP12 protein in both of the gene deletion strains. By Western analysis, we were able to detect a band of approximately 12 kDa (determined by comigration with appropriate size markers [data not shown]) that was not detected in the deletion strains (Fig. 6D). In the same Western blot, extracts from an *S. cerevisiae* wild-type strain (JK9-3da) and an *fpr1Δ* strain were included as positive controls for the anti-FKBP12 serum used (Fig. 6D).

After both *fkbA* deletion strains were rigorously verified, spores from each strain were germinated on YPD agar medium supplemented with rapamycin and FK506 in parallel with spores from the parental background strains, i.e., R7B and MU402 (MU402 was derived from R7B). While the growth of each parental strain

was sensitive to rapamycin and FK506, growth of both *fkbA* mutant strains was unaffected in the presence of either drug. All strains were sensitive to growth inhibition in the presence of cyclosporine (Fig. 6E). Taken together, these results confirm that FKBP12, encoded by the *fkbA* gene, functions as the rapamycin/FK506 receptor in *M. circinelloides*.

Rapamycin improves survival of *Galleria mellonella* larvae infected with a lethal dose of *M. circinelloides* spores. Clinical treatment of human-invasive fungal infections caused by zygomycete (*Rhizopus* and *Mucor*) species is notoriously difficult to achieve due to the intrinsic resistance of most zygomycete species to the current antifungal drug armamentarium (reviewed in references 44 and 50). In light of the inhibitory effects of rapamycin on *M. circinelloides* growth, we tested whether targeting the Tor pathway with rapamycin could serve as a potential antifungal therapeutic approach. To this end, we used larvae from the wax moth *Galleria mellonella* infected with *M. circinelloides* spores as an invertebrate model of disseminated infection. The use of *G. mellonella* as a model of infection is becoming increasingly popular due to its ease of use and high correlation with murine models of disseminated infection (9, 12). Healthy larvae are light colored and active, while larvae killed by a fungal infection are dark and immobile, facilitating scoring of mortality during assays.

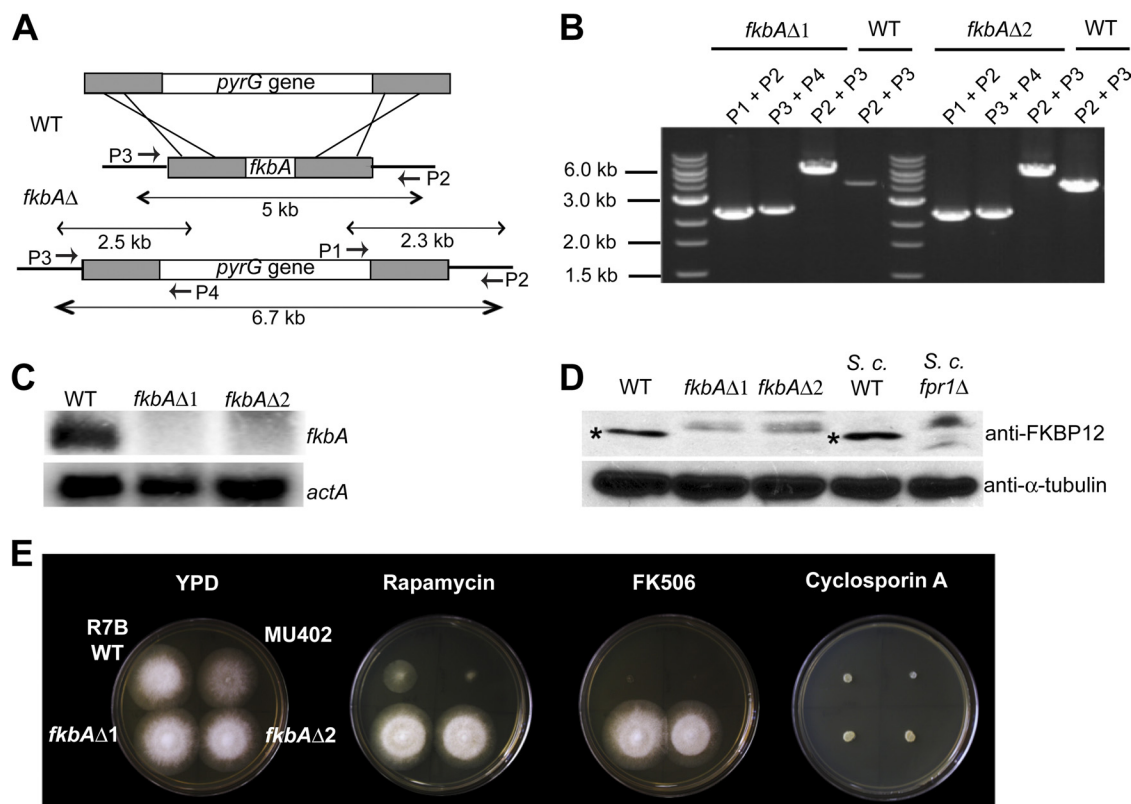


FIG 6 Targeted disruption of the *fkbA* locus confers rapamycin and FK506 resistance. (A) Schematic representation of genotyping primers used to verify disruption of the *fkbA* locus. (B) PCR genotyping of two independent *fkbA*Δ deletion strains. (C) Northern analysis of *fkbA* expression in the wild-type parental strain (MU402) and the two independent *fkbA*Δ strains. (D) Western analysis of FKBP12 protein expression in the wild-type parental strain (MU402) and the two independent *fkbA*Δ strains. Extracts from each sibling strain were used for Western analysis. FKBP12 protein was detected with antiserum raised against *S. cerevisiae* FKBP12. Alpha-tubulin (loading control) was detected using a monoclonal antibody raised against *S. cerevisiae* alpha-tubulin. (E) Spore suspensions from the parental strains R7B (parent of MU402) and MU402 and from the *fkbA*Δ strains were spotted on YPD agar and YPD agar supplemented with 100 nM rapamycin, 1 μg/ml FK506, or 100 μg/ml cyclosporine (cyclosporin A) and were incubated for 48 h at room temperature.

In this model of infection, injection of control PBS had no effect on survival of larvae (Fig. 7). Injection of 500 spores of the *M. circinelloides* R7B strain caused 100% death within 5 days after infection, while spores from the *M. circinelloides* NRRL3631 strain were aviru-

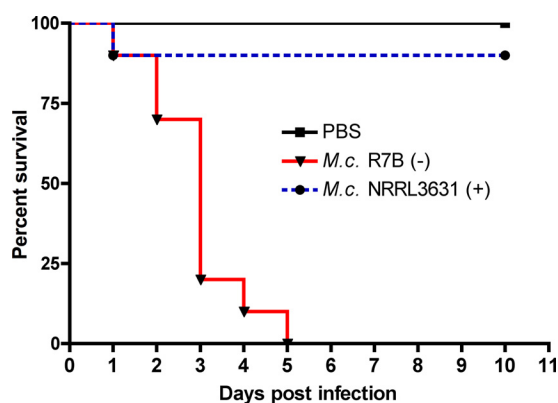


FIG 7 Infection by *M. circinelloides* R7B spores results in acute mortality of *G. mellonella* larvae. Thirty larvae per treatment were infected with 500 *M. circinelloides* spores from the R7B and NRRL3631 wild-type strains. Infecting *G. mellonella* with *M. circinelloides* R7B spores resulted in acute mortality rates, while infections by *M. circinelloides* NRRL3631 spores did not.

lent in *G. mellonella* (Fig. 7), consistent with recent published results (31), and were not used in subsequent experiments.

Treatment of *G. mellonella* infected with *M. circinelloides* R7B spores with a dose of 33 mg of rapamycin/kg resulted in a 50% survival rate, a statistically significant ($P > 0.0133$; log rank test) improvement in survival compared to the 0% survival rate of PBS-treated infected controls (Fig. 8). Similar rapamycin treatment of *G. mellonella* larvae infected with spores from two independently derived R7B *fkbA* deletion strains (RBM1 and RBM2) did not have any effect on its virulence, indicating that inhibition of *McTor* by rapamycin is protective in *G. mellonella*. Notably, treatment of uninfected larvae with the same dose of rapamycin had a negligible effect on their survival in relation to PBS controls, indicating that rapamycin's known immunosuppressive effect in humans has a negligible impact on *G. mellonella* survival (Fig. 8).

Surprisingly, and contrary to our *in vitro* results, monotherapy with FK506 did not improve survival of *G. mellonella* larvae infected with R7B spores at the dose administered (0.13 mg FK506/kg) (data not shown). This dose followed the MIC that elicited a growth inhibitory effect in our *in vitro* assays. However, FK506 proved not to be beneficial in the *in vivo* model of infection, possibly due to a reduced stability of FK506 in *G. mellonella*, higher rates of drug clearance, or competition for drug binding by endogenous FKBP12.

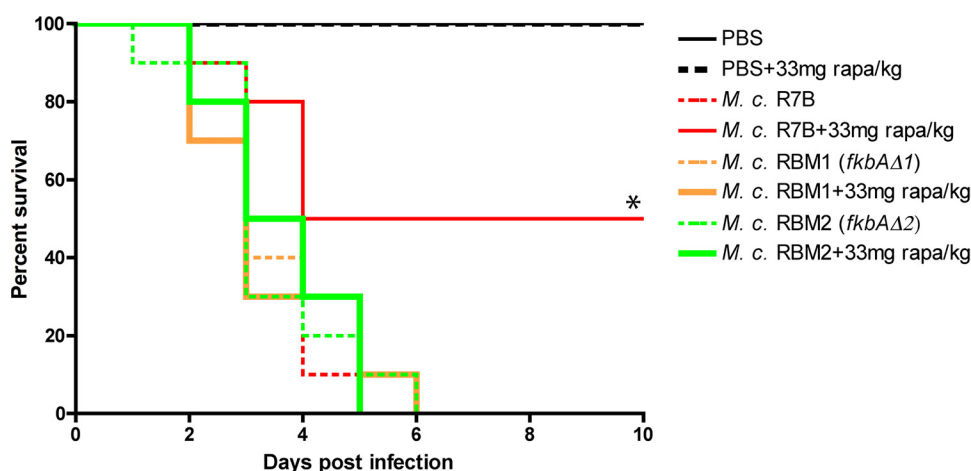


FIG 8 Pharmacological inhibition of Tor enhances survival of *Galleria mellonella* infected with a lethal dose of *M. circinelloides* spores. Inhibition of *McTor* by rapamycin exhibits a therapeutic benefit in the *G. mellonella* model of *M. circinelloides* pathogenesis. Ten larvae per group were infected with PBS alone, rapamycin alone, or 500 spores of *M. circinelloides* R7B or the *fkbA*Δ strain RBM1 or RBM2. Larvae were treated with PBS or rapamycin (33 mg rapamycin/kg of body weight) at 4 h postinfection and grown at room temperature, and viability was scored daily. An asterisk denotes a statistically significant change in survival of infected *G. mellonella* larvae after treatment with rapamycin in relation to untreated larvae. Rapamycin was not beneficial to larvae infected with spores from the *fkbA*Δ strains RBM1 and RBM2. Survival curves are representative of two independent experiments.

Overall, the therapeutic benefit imparted by targeting the Tor pathway during infection of an invertebrate model of *M. circinelloides* dissemination establishes the Tor pathway as an attractive target for the development of antifungal therapy against life-threatening zygomycosis.

DISCUSSION

The Tor signaling pathway serves as an attractive target for antifungal therapy due to its central role in regulating fungal growth and its broad conservation among species in the fungal kingdom, with the notable exception of the Microsporidia (28, 47). The Tor signaling cascade evolved prior to the last common ancestor to the metazoan and fungal lineages, in accord with its known conservation in metazoans, land plants, algae, and fungi (2, 47). In accord with the deep ancestral roots of the Tor pathway, comparative genome analyses across multiple species throughout the fungal kingdom have revealed a remarkable conservation in the amino acid sequences of Tor homologs. Of particular interest are the invariably conserved residues within the hydrophobic pocket of the FRB domain that are critical for rapamycin binding to Tor (5, 7, 34, 47, 52, 59). This observation is in agreement with previous studies showing that rapamycin has broad-spectrum antifungal activity against several human pathogens, including *Candida albicans* and *Cryptococcus neoformans*, and that its activity in these species is mediated by conserved rapamycin-FKBP12 drug-protein complexes (1, 13, 15, 17, 56, 58).

In this study, we further demonstrate that rapamycin inhibits growth of the zygomycetes *P. blakesleeanus*, *R. oryzae*, and *M. circinelloides*, demonstrating a conserved role for Tor in regulating the growth of basal fungal pathogens (Fig. 1 and Tables 2 and 3). We also established that rapamycin's antifungal action is mediated via conserved FKBP12-dependent inhibition of a Tor kinase homolog in *M. circinelloides*. First, expression of *McFKBP12* in an *S. cerevisiae* FKBP12-deficient strain restored rapamycin sensitivity (Fig. 4A). In addition, overexpression of the *M. circinelloides* Tor FRB domain rescued rapamycin toxicity in an *S. cerevisiae* wild-type strain (Fig. 4B). Rapamycin also promoted interactions

between *McFKBP12* and the *McTor* FRB domain in the yeast two-hybrid assay (Fig. 4C). These lines of evidence demonstrate a conserved mechanism of FKBP12-mediated inhibition of Tor by rapamycin in a zygomycete fungal pathogen.

Second, we isolated a spontaneously rapamycin-FK506 doubly resistant mutant harboring a mutation in the acceptor splice site of intron 2 of *fkbA* that abrogates FKBP12 protein expression (Fig. 5A to C), presumably by nonsense-mediated decay. A second independent mutant, in which leucine 91 (a conserved calcineurin A binding residue in FKBP12) is replaced by proline, while sensitive to rapamycin, displayed FK506 resistance (Fig. 5D). In the crystal structure of the human FKBP12-FK506-calcineurin ternary complex, isoleucine 90 (I90) (corresponding to leucine 91 in *M. circinelloides* FKBP12) changes conformation in the ternary complex and occupies a hydrophobic pocket in the calcineurin A subunit (21). Futer et al. identified I90 as a constituent of the human FKBP12 (hFKBP12) 80s loop, a critical composite surface essential for calcineurin binding (19). Replacing I90 with a polar amino acid such as lysine decreases the affinity of the hFKBP12-FK506 binary complex for calcineurin 26,000-fold (wild-type K_i , 5.5 nM; I90K mutant K_i , 14,300 nM), without significantly affecting the binding affinity for either FK506 or rapamycin (19). Similarly, in SM4, the L91P substitution leads to a shorter cyclic side chain that would predictably be occluded from the calcineurin A hydrophobic pocket and thereby diminish calcineurin binding in the presence of FK506. Third, ablation of the *fkbA* locus by gene replacement conferred both rapamycin and FK506 resistance (Fig. 6), further demonstrating a role for FKBP12 in mediating rapamycin and FK506 antifungal action. Taken together, these studies further demonstrate that the widespread antifungal activity of rapamycin and its mechanism of action are conserved in the ascomycetes *S. cerevisiae* and *C. albicans*, the basidiomycete *C. neoformans*, and the zygomycete pathogen *M. circinelloides* (13, 15, 17, 23).

Our studies also demonstrate that in a *G. mellonella* model of infection, rapamycin protects 50% of larvae against a lethal infection by *M. circinelloides* (Fig. 8), providing a key proof of principle

that targeting the Tor pathway with inhibitors could potentially be used during antifungal therapy against zygomycetes. These findings are in agreement with studies showing that rapamycin treatment protected 50% of mice from an otherwise lethal infection with *C. albicans* and improved survival of mice with invasive aspergillosis (1, 26). Given that patients undergoing solid organ transplants are exceedingly susceptible to infections by zygomycetes, our findings suggest that patients receiving rapamycin immunosuppressive therapy may also benefit from its antifungal activities against infections by zygomycete pathogens. Indeed, studies have shown that solid organ transplant patients immunosuppressed with tacrolimus (FK506) have lower mortality rates for both cryptococcosis and zygomycosis (48, 49), warranting similar studies with patients immunosuppressed with rapamycin.

While the immunosuppressive effects of rapamycin currently outweigh its antifungal action, one report suggests that rapamycin in limited doses can synergize with antifungal agents against zygomycetes, providing a scenario in which rapamycin's antifungal action might have therapeutic value. In one study (16), rapamycin was shown to exhibit synergistic activity with amphotericin B and posaconazole against several zygomycete clinical isolates, and it showed antifungal activity on its own against *Rhizopus microsporus*, *M. circinelloides*, *Rhizomucor pusillus*, and *Mycoclados corymbiferus*. These results and our findings reported here provide support for harnessing the antifungal activity of rapamycin in combination with antifungals that are less effective against zygomycetes on their own.

Combinatorial therapy resulting in simultaneous inhibition of multiple pathways enhances the efficacy of individual drugs by broadening potency and decreasing the emergence of drug resistance. The challenge remains to exploit such combinatorial therapy while avoiding rapamycin's immunosuppressive effects. Advances in this regard are under way with the development of less immunosuppressive rapamycin analogs with potent antifungal effects (14, 15, 38). Alternatively, possible clinical applications involve topical formulations, which circumvent the immunosuppressive effects, and the use of lipid formulations. Indeed, rapamycin encapsulated within PEG-DSPE (polyethylene glycol-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy) micelles retains potent *in vitro* activity against *C. albicans* (54). Lipid formulations could also offer the advantage of masking immunosuppressive effects by allowing localized delivery of these drugs to sites of infections while also lowering effective doses and toxic side effects. Lipid formulations of rapamycin could potentially be beneficial in combinatorial therapy with lipid formulations of amphotericin B, the recommended course of treatment against zygomycosis (44, 51, 53). Hsp90 is also an attractive pathway for antifungal intervention in conjunction with Tor inhibitors, since Hsp90 inhibitors have robust antifungal activity against several fungal pathogens (12). Like the case with rapamycin, the challenge remains to develop specific inhibitors capable of discriminating pathogen protein chaperones from those of the host.

In conclusion, our studies have defined an evolutionarily conserved mechanism of rapamycin action in a basal fungal pathogen, a theme that is emerging as a broadly conserved paradigm in the fungal kingdom. It now remains to be tested whether rapamycin and less immunosuppressive analogs can be beneficial in vertebrate animal models of zygomycosis.

ACKNOWLEDGMENTS

We thank Santiago R. Torres-Martinez for providing strains, protocols, and valuable discussions.

This study was supported by NIH R01 grants CA154499 (to M.E.C.) and A150438 (to J.H. and M.E.C.) and by American Heart Association award 1OPRE3310015 (to C.A.S.).

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